

# Control of Mammary Epithelial Differentiation: Basement Membrane Induces Tissue-specific Gene Expression in the Absence of Cell-Cell Interaction and Morphological Polarity

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**Abstract.** Functional differentiation in mammary epithelia requires specific hormones and local environmental signals. The latter are provided both by extracellular matrix and by communication with adjacent cells, their action being intricately connected in what appears to be a cascade of events leading to milk production. To distinguish between the influence of basement membrane and that of cell-cell contact in this process, we developed a novel suspension culture assay in which mammary epithelial cells were embedded inside physiological substrata. Single cells, separated from each other, were able to assimilate information from a laminin-rich basement membrane substratum and were induced to express  $\beta$ -casein. In contrast, a stromal environment of collagen I was not sufficient to

induce milk synthesis unless accompanied by cell-cell contact. The expression of milk proteins did not depend on morphological polarity since E-cadherin and  $\alpha_6$  integrin were distributed evenly around the surface of single cells. In medium containing  $5 \mu\text{M}$   $\text{Ca}^{2+}$ , cell-cell interactions were impaired in small clusters and E-cadherin was not detected at the cell surface, yet many cells were still able to produce  $\beta$ -casein. Within the basement membrane substratum, signal transfer appeared to be mediated through integrins since a function-blocking anti-integrin antibody severely diminished the ability of suspension-cultured cells to synthesize  $\beta$ -casein. These results provide evidence for a central role of basement membrane in the induction of tissue-specific gene expression.

**R**EGULATION of differentiation in complex tissues is determined not only by growth factors and hormones, but also by intercellular communication and by interactions between cells and their extracellular matrix (Stoker et al., 1990). In epithelial tissue, part of the extracellular matrix (ECM)<sup>1</sup> occurs in the form of a basement membrane, which provides positional information for cells and cues for organizing intracellular structure, as well as signals that regulate cellular behaviour. An ideal model for studying how ECM signals are transduced to control tissue-specific function is the simple epithelium of mammary gland. In adult animals, the cells undergo developmental changes during pregnancy and become highly secretory at lactation. The great advantage of this system is that in culture, mammary epithelial cells regain their differentiated phenotype only under suitable hormonal and substratum conditions. The model can therefore be used to understand the mechanism by which tissue-specific genes are expressed (Bissell and Hall, 1987; Streuli and Bissell, 1991).

Considerable evidence now indicates that basement membrane plays a significant role in regulating mammary phenotype. In vivo, the alveolar epithelium of rodent mammary

gland is surrounded by a basement membrane; its dissolution during involution correlates with functional regression of the gland (Talhouk et al., 1991). In culture, on stromal collagen I substrata, mammary cells synthesize and secrete milk proteins only if an endogenous basement membrane is deposited basally to the cell layer; this occurs if collagen I gels are released into the culture medium (Streuli and Bissell, 1990). More complete differentiation can be achieved on the basement membrane matrix derived from Engelbreth-Holm-Swarm tumour (EHS matrix, or 'matrigel') where, in addition to high level milk production, the cells undergo morphogenetic changes and form spherical structures that resemble the alveoli of lactating mammary gland with striking fidelity (Li et al., 1987; Barcellos-Hoff et al., 1989; Chen and Bissell, 1989; Aggeler et al., 1991). However, under conditions where basement membranes are not present, such as on nonreleased collagen gels or on plastic dishes, mammary epithelial cells express little or no milk proteins even when cultured with lactogenic hormones.

The inability of mammary epithelium to function correctly in the absence of basement membrane strengthens the argument for its involvement in the induction and maintenance of differentiation (Bissell and Barcellos-Hoff, 1987; Streuli and Bissell, 1991). However, biochemical function in cells cultured on either floating collagen gels or basement

1. *Abbreviations used in this paper:* DAPI, 4,6-diamidino-2-phenylindole; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm.

membrane substrata is accompanied by cytostructural alterations and by increased interactions between adjacent cells. Because such interactions might influence tissue-specific gene expression, it has now become crucial to address the issue of whether these local environmental cues are indeed required for mammary cell differentiation.

We have therefore developed a novel suspension culture assay, firstly to distinguish between the influence of basement membrane and that of cell-cell contact in the control of mammary phenotype, and secondly to identify whether or not basement membrane can signal changes in gene expression via integrins. Here we present evidence that basement membrane regulates the synthesis of milk proteins in mammary epithelium directly, and that integrins are involved in this process. We also discuss the contribution of cell-cell interactions, and suggest that these signals have additional roles in the differentiation of a functional epithelium.

## Materials and Methods

### Substrata and Antibodies

EHS matrix was prepared from EHS tumors passaged in C57BL mice by urea extraction at 4°C (Kleinman et al., 1986). This material was stored for up to four weeks at 0°C. Each preparation contained 5–10 mg per ml protein, and was routinely assayed for purity and lack of protein degradation on 5% reducing SDS-polyacrylamide gels followed by silver staining. Growth factor-depleted EHS matrix was prepared from freshly made EHS matrix by precipitation twice with 20% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> on ice followed by dialysis, using the protocol of Taub et al. (1990), and was used immediately. Collagen I was prepared from rat tails (Lee et al., 1984) and was stored at a concentration of 2–3 mg per ml in 0.1% acetic acid at 4°C. For use in the suspension assay, it was dialyzed against three changes of water (total time 48 h, 4°C) and adjusted to 1 × F12 medium immediately before use. Under these conditions, toxicity due to salt and pH imbalance was minimized, and the collagen solution remained workable for two to three hours. To form base layers for either the primary epithelial cultures or for the cell-embedded gels, the substrata were spread at 15.6 μl per cm<sup>2</sup>, and gelled at 37°C.

Polyclonal rabbit antiserum to mouse milk was obtained by immunization with skimmed milk proteins as previously described (Lee et al., 1985). A mouse mAb to rat β-casein was a gift from Dr. C. Kaetzel (Institute of Pathology, Case Western Reserve University, Cleveland, OH) (Kaetzel and Ray, 1984). The rat mAb to α<sub>6</sub> integrin (GoH3; ascites) was a gift from Dr. A. Sonnenberg (Lab. for Experimental and Clinical Immunology, University of Amsterdam, The Netherlands) (Sonnenberg et al., 1987). The rat mAbs to E-cadherin (ECCD-1, ECCD-2; culture supernatant) were generously supplied by Dr. M. Takeichi, Dept. Biophysics, Kyoto University, Japan (Yoshida-Noro et al., 1984; Shirayoshi et al., 1986). The rat mAb to β<sub>4</sub> integrin (346-11A) was a gift from Dr. S. Kennel (Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN) (Kennel et al., 1989). Polyclonal goat antiserum to baby hamster kidney fibroblast β<sub>1</sub> integrins (anti ECM-R) was the kind gift of Dr. C. Damsky (Dept. Anatomy and Stromatology, University of California, San Francisco, CA) (Knudsen et al., 1981; Damsky et al., 1982). Polyclonal rabbit antiserum to mouse laminin was obtained from E. Y. Labs (San Mateo, CA).

### Cell Culture

Primary mammary epithelia were prepared from 14.5-d pregnant CD-1 mice and cultured on physiological substrata as described previously (Lee et al., 1985; Barcellos-Hoff et al., 1989) in the presence of lactogenic hormones and, for the first 36 h, 1 mg per ml fetuin (Sigma Chemical Co., St. Louis, MO) and 10% FCS. The initial plating density was 4.7 × 10<sup>5</sup> cells per cm<sup>2</sup>. Insulin (5 μg/ml) and lactogenic hormones (1 μg/ml hydrocortisone, 3 μg/ml prolactin) were included in all experiments, and the medium was changed every day. The secondary cultures were derived from primaries cultured for five or six days on plastic dishes. These essentially nonfunctional cells were also cultured with lactogenic hormones. Monolayers were washed briefly with STV (0.05% trypsin, 0.02% EDTA in saline A) and trypsinized in STV for 10–15 min, at which time serum

was added to inactivate the enzyme. In the serum-free experiments, trypsinization was stopped with 5 mg per ml soybean trypsin inhibitor (Gibco Laboratories, Grand Island, NY). The cells were washed with F12 medium and counted. Mild trituration broke up large cell aggregates and resulted in populations containing 55–65% single cells.

Secondary cultures were plated at 4.7 × 10<sup>5</sup> cells per cm<sup>2</sup> onto various substrata using identical conditions to those for the primaries. For the suspension assays, cells were embedded inside physiological substrata, such as the EHS matrix or a collagen I gel. Freshly harvested cells (10<sup>6</sup>) were pelleted for 2 min at 1,300 g in sterile microfuge tubes, chilled on ice, immediately resuspended with mild trituration to a density of 2.2–4.5 × 10<sup>6</sup> cells per cm<sup>3</sup> in EHS matrix or collagen I at 0°C, and 100-μl aliquots were plated onto preformed base layers of the corresponding substratum. At the latter density the cells were well separated from each other, being on average at least five cell diameters apart. Some cells reaggregated immediately after trypsinization and remained as clusters during the course of the experiment. The cell-containing substrata were gelled (30–60 min, 37°C) and then covered with hormone-containing medium. After six days in culture the majority of cells were still isolated from each other; at six days, 44–60% of all the single cells plus clusters were single, 16–31% were doublets, and the remainder existed in larger clusters. Cells induced to differentiate were detected by immunohistochemistry, either directly in the whole mounts, or in cryosectioned cultures. Cell viability was confirmed by autoradiography performed on sections of suspension cultures that had been pulse chased with <sup>35</sup>S]methionine before fixation.

In some experiments, heat-inactivated (1 h, 56°C) anti-ECM-R antibody was included in the substratum (1%) and in the culture medium (0.5%). At a similar concentration (0.6–1%), this antibody blocked B16-BL6 melanoma cell adhesion to basement membrane matrices (Kramer et al., 1990), and blastocyst outgrowth on laminin and fibronectin (Sutherland et al., 1988). It also blocked adhesion of PC12 cells to both laminin and collagen IV substrata, and prevented outgrowth of their neuronal processes on laminin (Tomaselli et al., 1987). In addition, the binding of mouse mammary cells to laminin-coated dishes was inhibited efficiently by the anti ECM-R antibody (not shown).

### Immunohistochemistry

Immunostaining was carried out according to general principles in Harlow and Lane (1988). Non-specific antibody-binding sites were blocked and all washes were done in PBS containing 0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20, 0.05% NaN<sub>3</sub> (at least 1 h, room temperature). Sections or whole mounts were incubated with primary antibodies for 60 min and subsequent antibodies for 30 min. Primary rat antibodies were detected with Texas red anti-rat IgG (Caltag Labs, South San Francisco, CA), mouse antibodies with either FITC anti-mouse IgG (Amersham Corp.), or biotinylated anti-mouse IgG (Amersham Corp.) followed by Texas red streptavidin (Amersham Corp.), and rabbit antibodies were detected with biotinylated anti-rabbit IgG (Amersham Corp.) followed by Texas red streptavidin. In all experiments nuclei were counter stained (3 min) during the final wash with 0.5 μg per ml DAPI (4,6-diamidino-2-phenylindole; Sigma Chemical Co.). The fluorescence optics were Zeiss (Oberkochen, Germany), and photography was with EL400 or TX400 film (Eastman Kodak Co., Rochester, NY).

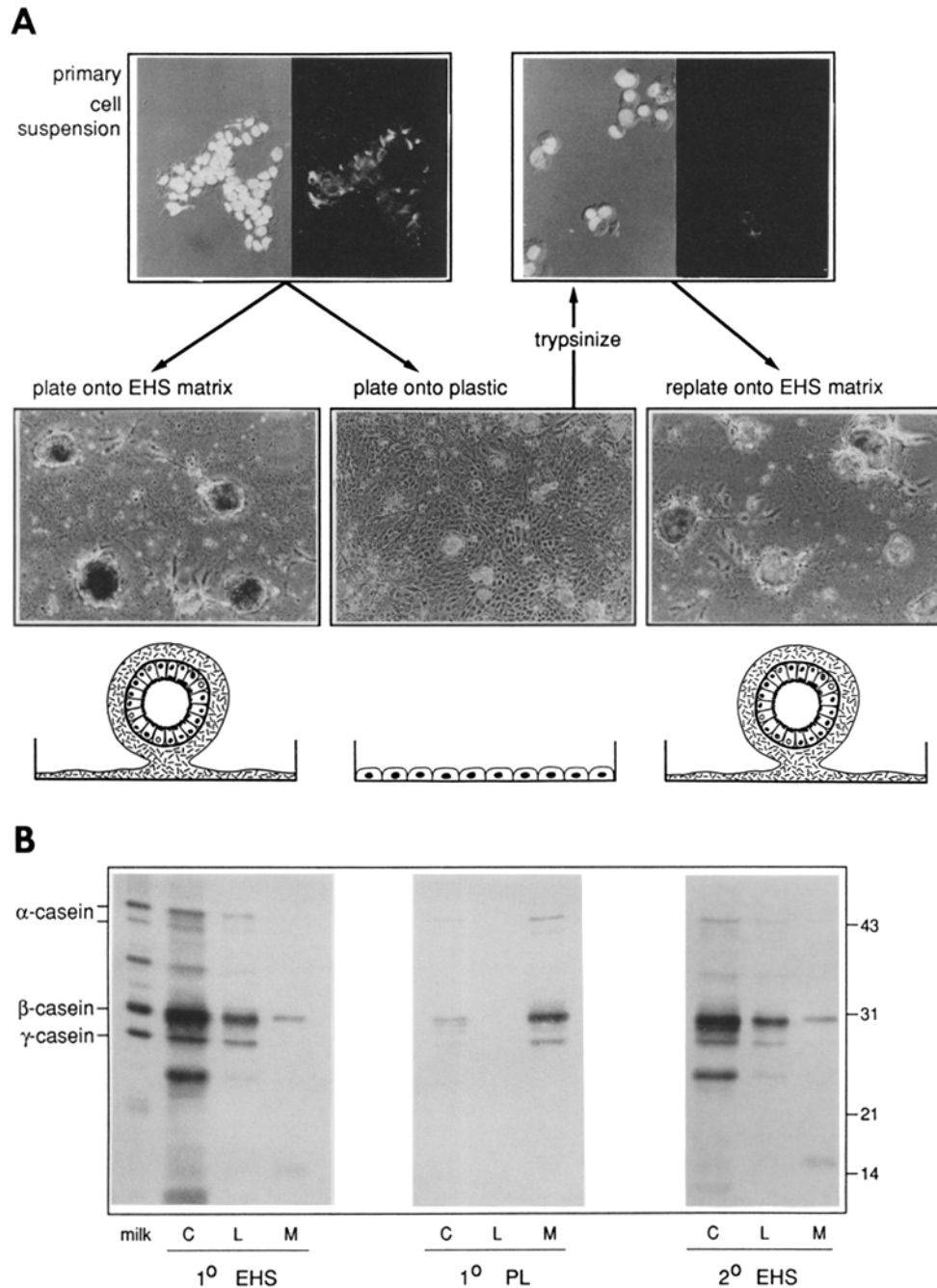
For the whole mounts, cells were cultured inside the relevant substratum on coverslips, fixed with 2% paraformaldehyde in PBS (20 min, room temperature), quenched with 0.1 M glycine (3 × 20 min), the cell membranes permeabilized in 1% Triton X-100 (90 s), and the cells stained with the β-casein mAb. All other suspension cultures were fixed with 2% paraformaldehyde, quenched with 0.1 M glycine, and equilibrated first with sucrose and subsequently with Tissue-Tek OCT compound (Miles Scientific Div., Elkhart, IN), before freezing on a dry ice/ethanol bath. Sections were cut with a cryotome (E. Leitz, Inc., Rockleigh, NJ), collected on gelatin-coated coverslips and air dried before immunostaining with the relevant antibody. To reduce nonspecific background, the detection antibodies were adsorbed onto a thin layer of gelled EHS matrix before use (60 min, 37°C). The counting procedure for β-casein-staining cells was as follows. Single cells or cell clusters were located with Nomarski optics in conjunction with DAPI fluorescence, nuclei then were viewed with fluorescence alone to count the number of cells in the cluster, and the single cells or clusters were finally scored for casein expression using the FITC or Texas red fluorescence. Optical sectioning through the thick sections or whole mounts ensured that the scored single cells were actually single. Although some clusters expressing β-casein contained a few nonstaining cells, such clusters were scored as being positive. In most experiments, enough fields were viewed to score more than 200 single cells plus clusters.

Alveolar cultures of mammary cells on EHS matrix were fixed with paraformaldehyde, quenched, and embedded in OCT before freezing and cryosectioning. For this purpose, 4- $\mu$ m sections were cut at an angle oblique to the plane of the culture dish. Monolayer cultures, grown on acid-washed glass coverslips, were stained after a 5 min methanol/acetone (1:1) fixation at  $-20^{\circ}\text{C}$ .

### Protein Analyses

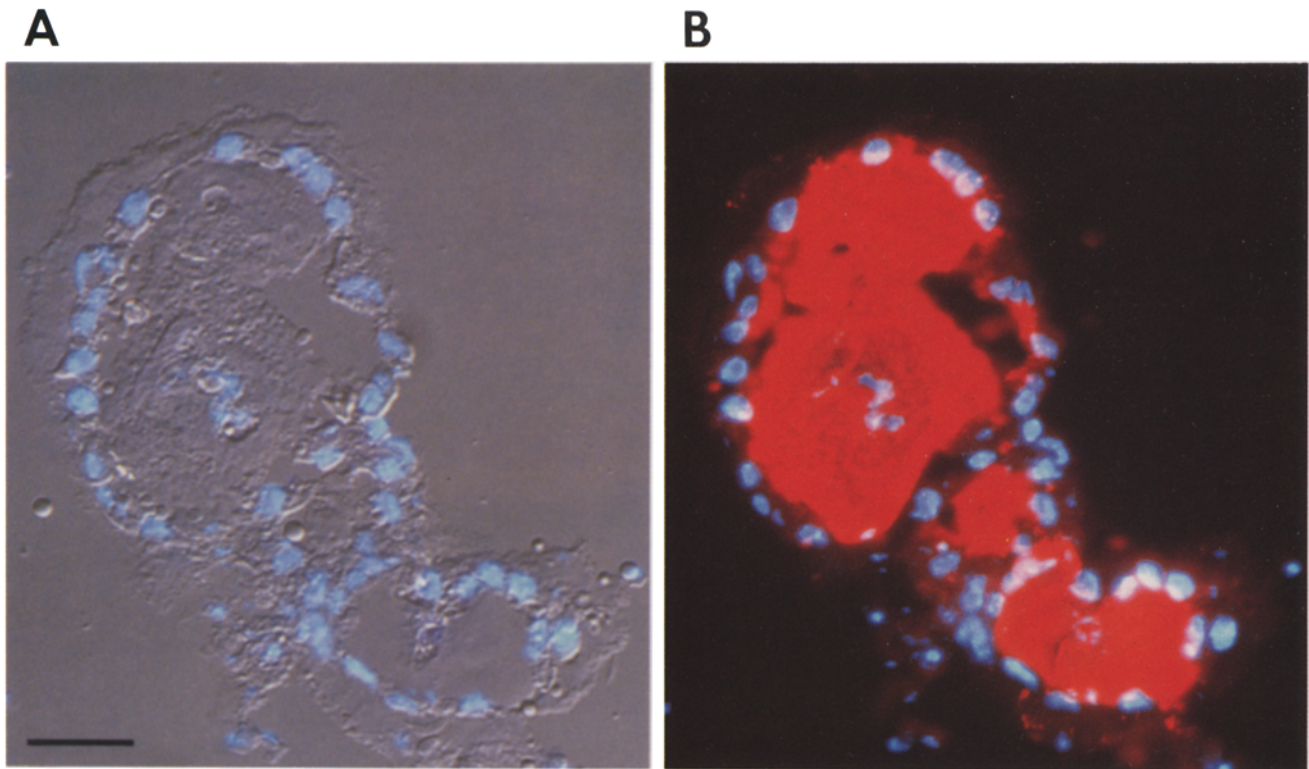
For immunoblotting, cells were harvested 24 h after the previous media change. Media fractions were collected and cleared (1 min, 16,000 g). Luminal fractions, containing material secreted inside the cultured alveoli, were extracted with 2.5 mM EGTA as described (Barcellos-Hoff et al., 1989) and cleared. These fractions were then diluted in sample buffer (10% glycerol, 50 mM Tris-HCl, 2% SDS, 0.02% bromophenol blue, 5% mercaptoethanol, pH 6.8). The remaining cell fractions were scraped directly

into sample buffer. The samples from different culture conditions were normalized by counting cells (in triplicate) on identical dishes, and adjusting each of the media, luminal, and cell fractions to an equivalent volume. Material from only  $\sim 1,000$  cells was resolved on 0.75-mm-thick 13% polyacrylamide gels under reducing conditions, and transferred to Immobilon-P membranes (Millipore Continental Water Systems, Bedford, MA) using a dry blot apparatus. The membranes were incubated overnight in a wash buffer containing 100 mM Tris-HCl, 150 mM NaCl, 0.3% Tween 20, the nonspecific sites blocked in the same buffer containing 2% BSA, and the milk proteins detected with rabbit anti-mouse milk antiserum, followed by alkaline phosphatase-conjugated anti-rabbit IgG (Caltag Labs), and subsequently BCIP/NBT (bromo chloro indolyl phosphate/nitro blue tetrazolium; Sigma Chemical Co.). Mouse milk (50 ng) was routinely included in the gels as a control. Biotinylated size markers were detected with alkaline phosphatase conjugated streptavidin (Zymed).



**Figure 1.** Single mammary cells can redifferentiate on a basement membrane matrix. (A) Cells isolated from pregnant mammary gland existed as aggregates, and many cells ( $\sim 70\%$ ) expressed milk proteins, here assayed by immunofluorescence of cytopins using mAb specific for  $\beta$ -casein (top left, Nomarski images of the cells are also shown, their nuclei stained with DAPI). After six days in culture on EHS matrix, they formed spherical structures (shown by phase-contrast microscopy and as a schematic cross section). On a plastic substratum, a squamous monolayer resulted. These cells could be trypsinized to single cell suspensions (top right), of which only 6–17% express  $\beta$ -casein in cytospin analysis; in this field a small cluster of three cells was weakly positive. The cells aggregated and formed alveolar, casein-expressing spherical structures when replated on EHS matrix and cultured for six days (right panels). (B) Secondary cultures ( $2^{\circ}$ ) maintained for six days on EHS matrix (EHS) accumulated quantities of milk comparable to primary cultures ( $1^{\circ}$ ). This immunoblot shows caseins in the cell fractions (C) and those secreted into lumina (L) of spheres. The high levels in the former were largely due to precipitation of secreted milk within the lumina and its consequent inefficient extraction. Very small amounts of milk were secreted basally into the medium (M) of these cultures. Cells cultured on plastic dishes (PL) produced low overall levels of milk; what is seen was due to synthesis by cells remaining in aggregates (unpublished data). In each case, material from  $\sim 1,000$  cells was

resolved by SDS-PAGE; note that the level of milk production by only this number of cells cultured on the EHS matrix exceeded 50 ng of mouse milk. Size markers (in kD) are indicated.



**Figure 2.**  $\beta$ -casein accumulates in the lumina of alveolar cultures. 4- $\mu$ m sections of six day EHS matrix cultures were stained by immunofluorescence using a mAb specific for  $\beta$ -casein. Views of DAPI-staining nuclei are superimposed either on (A) a Nomarski image of a cryosection, or on (B) an image of the  $\beta$ -casein localized by immunofluorescence. Bar, 30  $\mu$ m.

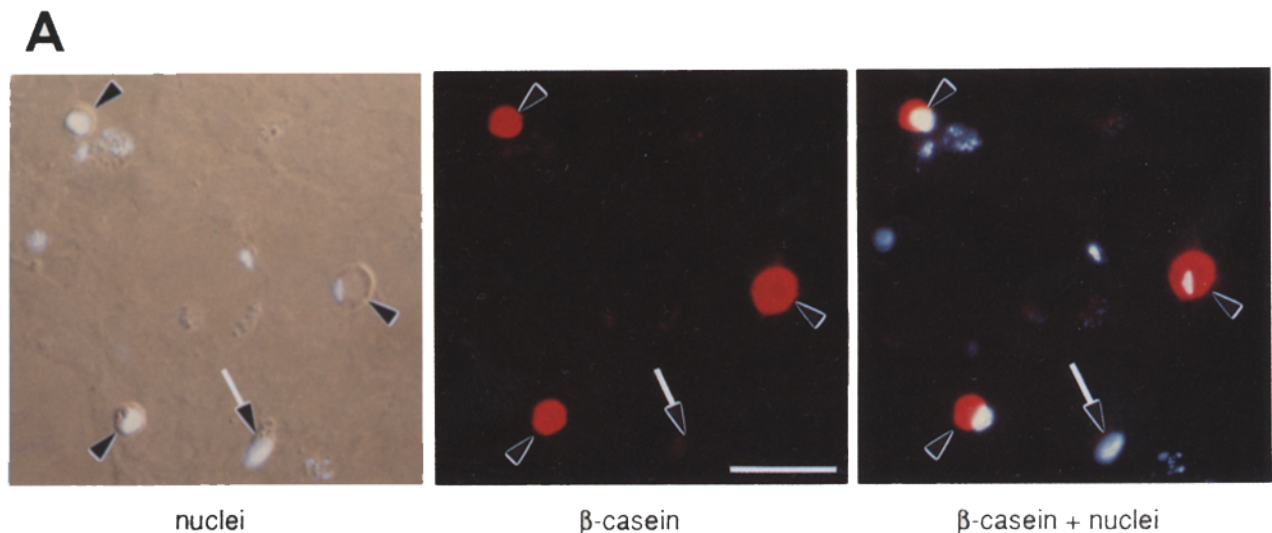
Immunoprecipitation was carried out as described previously (Streuli and Bissell, 1990), except that cells were labeled for 4 h with 0.25 mCi per ml Tran<sup>35</sup> S-label (ICN Biomedicals, Irvine, CA), and the proteins were precipitated with rabbit anti-mouse milk antiserum followed by protein A sepharose (Sigma Chemical Co.), before separating on 13% polyacrylamide gels under reducing conditions.

## Results

### Differentiation of Mammary Epithelial Cells in Culture

The initial aim of these studies was to see whether single

mammary epithelial cells, separated from each other, could be induced to synthesize milk proteins in suspension culture. First, however, it was necessary to show that single mammary cells would redifferentiate in culture if they were brought together under optimal conditions. Primary preparations of epithelial cells recovered from collagenase digests of midpregnant mouse mammary gland existed almost exclusively as aggregates (Fig. 1 A, top left). More vigorous cell-separation techniques resulted in unacceptable mortality. When plated on plastic dishes, the cell aggregates spread to form a squamous cobblestone monolayer (Fig. 1 A) and did not deposit their own basement membrane (Streuli and Bis-



**Figure 4.**



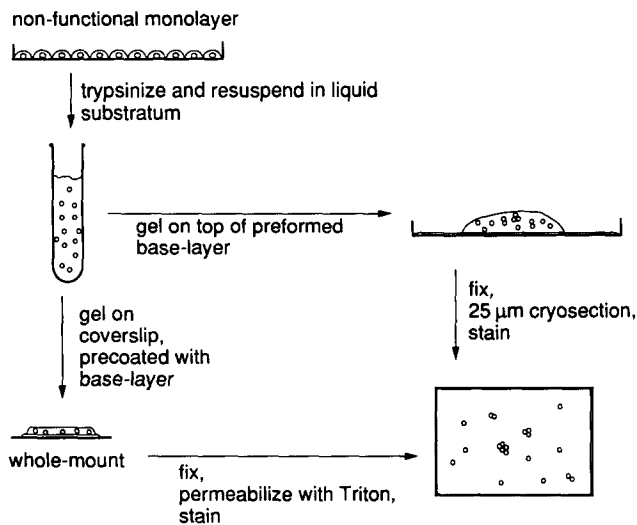


Figure 3.

sell, 1990). They were unable to synthesize milk proteins even in the presence of lactogenic hormones (Fig. 1 *A*, *top right*; and Fig. 1 *B*) (Lee et al., 1984; Li et al., 1987), and therefore represented a potentially useful starting cell population on which to test the effect of basement membrane. These cultures were easily trypsinizable, producing suspensions that contained mostly single cells (55–65%) as well as small clusters of two or more cells. When replated onto EHS

matrix, the cells eventually aggregated, underwent alveolar morphogenesis (Fig. 1 *A*), and secreted milk proteins vectorially (Figs. 1 *B* and 2). The extent to which differentiation could be reestablished compared well with that of primary cultures on the EHS matrix (Fig. 1 *B*).

### Differentiation Can Be Induced in Single Mammary Cells Cultured within Physiological Substrata

To ask whether mammary cells could differentiate in the absence of both cell–cell interactions and organized intracellular polarity, a single cell suspension assay was developed. For this purpose, cells were embedded inside either EHS matrix or a collagen I gel. The chemical properties of these substrata are ideal for maintaining cells in suspension culture, since they are viscous at 0°C and gel rapidly at 37°C to form malleable lattices with high-order supramolecular structures. Mammary epithelial cells, isolated from primary monolayers with low levels of function cultured on plastic dishes, were embedded so that they were well separated from one another at a cell density of  $2.2\text{--}4.5 \times 10^6$  cells per  $\text{cm}^3$ . Cell-containing droplets were allowed to gel on preformed base layers of either the corresponding substratum or collagen I, and were covered with growth medium (Fig. 3). Most cells cultured inside EHS matrix remained viable during the culture period. The cells were rounded, had smooth refractile borders, and their nuclei retained distinct nucleoli. Cells that became granular and had dispersed or absent nuclei were disregarded in subsequent analyses.

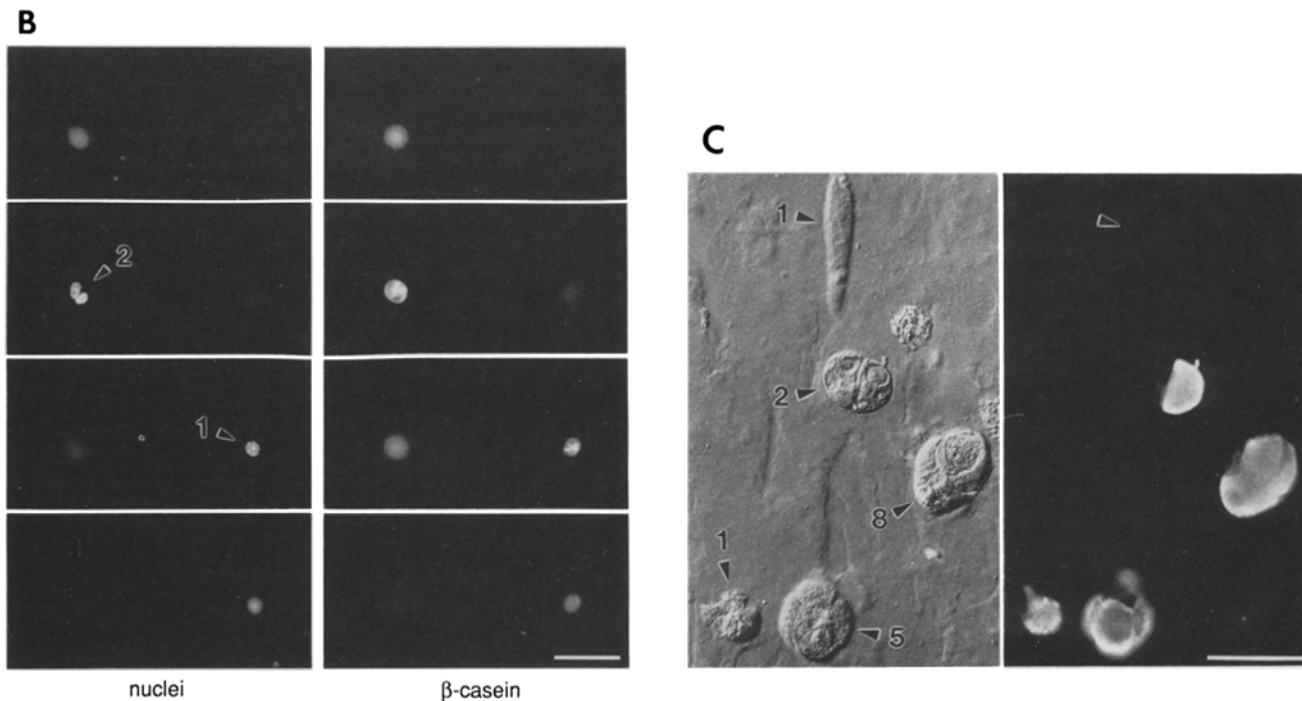


Figure 4. Mammary cells in EHS matrix suspension culture express  $\beta$ -casein. (A) Six day EHS matrix suspension cultures were stained by immunofluorescence using a  $\beta$ -casein-specific mAb. A cryosection shows three cells that strongly expressed  $\beta$ -casein (arrowheads), and one negative cell (arrow). The nuclei were counterstained with DAPI. (B) In a whole mount of cells embedded inside EHS matrix, one field of view was optically sectioned by photographing at different levels of focus. This type of analysis proves that single cells do indeed express  $\beta$ -casein. Here, one single cell and one cluster of two cells were both positive. (C) In this view of a thick 25- $\mu\text{m}$  cryosection, several clusters are visible. These clusters existed at the time of suspending the cells. By six days, the cells have coalesced and they produced high levels of casein. The number of nuclei in each cluster are indicated, and one nonstaining cell is shown (arrowhead). Bars: (A) 50  $\mu\text{m}$ ; (B) 50  $\mu\text{m}$ ; (C) 30  $\mu\text{m}$ .

**Table I.  $\beta$ -casein Expression in EHS Matrix Suspension Culture**

Experiment	Percentage of $\beta$ -casein staining			Total <sup>†</sup>
	Single cells	2 cell clusters	>4 cell clusters	
1	54 (307) <sup>‡</sup>	86 (102) <sup>‡</sup>	96 (94) <sup>‡</sup>	(590)
2	64 (133)	92 (39)	100 (14)	(223)
3	58 (342)	78 (127)	95 (86)	(679)
4	71 (98)	94 (34)	95 (22)	(180)
5 <sup>‡</sup>	55 (114)	72 (54)	98 (45)	(260)

Each experiment is from an independent culture derived from an independent preparation of primary mammary epithelial cells.

<sup>‡</sup> Cells cultured in whole mounts were analyzed in this experiment.

<sup>‡</sup> Numbers of single cells, 2 cell clusters, or >4 cell clusters counted.

<sup>†</sup> Total numbers of single cells + cell clusters counted; this includes the numbers of 3 and 4 cell clusters, which for the sake of clarity, are not shown in the table.

Assays for mammary function were carried out on individual cells in situ, using a mAb for  $\beta$ -casein as a suitable marker for biochemical differentiation (Kaetzel and Ray, 1984). In most experiments gels were sectioned, stained by indirect immunofluorescence, and counterstained with DAPI to localize nuclei.

Cryosections of embedded cultures revealed that single mammary cells within the EHS matrix could express  $\beta$ -casein (Fig. 4). In five independent experiments, 54–71% of the single cells were induced (Table I). Single cells were well represented within 25- $\mu$ m sections, which were approximately three times thicker than the average cell diameter of 10  $\mu$ m; occasionally, whole mounts were used to confirm that single cells were being studied (Fig. 4 B). The appearance of  $\beta$ -casein within cells was slightly punctate, but homogeneous. A sharp boundary of fluorescence existed at the cell periphery, suggesting that the casein was not secreted.

Small clusters of two or more cells also expressed  $\beta$ -casein; in some cases only one of a pair of cells was functional (Fig. 4 C). Large clusters stained strongly, and occasionally formed alveolar structures. In some of these it appeared that milk products were secreted vectorially into a luminal space (not shown), which may explain the greater intensity of fluorescence. The percentage of positive clusters was higher than that of single cells (Table I). However, since not every cell within each cluster stained for casein, this apparent enhancement of staining may have been due in part to random assortment of positive cells within the clusters.

Time course analysis was performed to confirm the inducibility of casein expression. The percentage of casein-positive cells rose dramatically during the first few days of culture (Table II), with the kinetics of induction resembling

**Table II. Induction of  $\beta$ -casein Expression in EHS Suspension Culture**

Day	Percentage of $\beta$ -casein staining			Total
	Single cells	2 cell clusters	>4 cell clusters	
1	4 (191)	11 (46)	48 (29)	(316)
2	15 (115)	34 (53)	84 (25)	(221)
4	34 (127)	86 (37)	94 (18)	(212)
6	63 (134)	89 (36)	100 (22)	(231)
9	60 (136)	81 (46)	95 (38)	(245)

the onset of function in alveolar cultures (Barcellos-Hoff et al., 1989; Aggeler et al., 1991). For single cells, biochemical differentiation required at least 1–2-d exposure to a basement membrane environment.

The requirement for a basement membrane was assessed by studying the behavior of mammary cells embedded within a collagen I gel. In this stromal environment, the cells responded very differently. Most single cells were unable to synthesize  $\beta$ -casein (Table III; Fig. 5 A). In contrast, many of the clusters were positive, showing that cell–cell interaction was necessary for functional differentiation when cells were embedded inside collagen I. However, since the positive cells, especially those in aggregates, were frequently associated with some laminin visible by immunofluorescence at the cell periphery (Fig. 5 B), the enhancement of  $\beta$ -casein expression was most probably because of an interaction with newly synthesized basement membrane components, as could be inferred from previous studies (Streuli and Bissell, 1990).

The ability of single cells to express  $\beta$ -casein when cultured inside EHS matrix implies that, as long as basement membrane components are present, cell–cell contact is not essential for biochemical differentiation. We then investigated whether or not morphological polarity was necessary for  $\beta$ -casein expression.

#### *The Expression of Milk Proteins Is Not Coupled to a Polarized Phenotype*

E-cadherin (uvomorulin) forms part of the epithelial intercellular adhesion system (Nagafuchi et al., 1987; Gumbiner et al., 1988; McNeill et al., 1990; Takeichi, 1991). This protein is located along lateral surfaces of cells in the highly polarized epithelium of mammary gland (not shown), a distribution that was also apparent between adjacent cells of “alveoli” produced in culture (Fig. 6 A). In suspension culture it was concentrated at sites of contact, indicating that in clusters the cells interacted closely with one another and established certain aspects of a polarized phenotype (Fig. 6 B). However in single cells, many of which were able to express  $\beta$ -casein, E-cadherin appeared in patches around the cell (Fig. 6, C and D), indicating a lack of morphological polarity.

To further rule out cell–cell interaction and polarity as mediators of milk protein expression, embedded cells were cultured in medium containing only 5  $\mu$ M  $Ca^{2+}$ . Such cells remained rounded and the clusters failed to coalesce. Neither the single cells nor the clusters showed any evidence of E-cadherin staining (Fig. 7 A). Yet, many of the same cells were still able to synthesize substantial levels of  $\beta$ -casein (Fig. 7 B). This applied both to secondary cultures and to CID-9 cells, a recently isolated mouse mammary cell strain that exhibits ECM-dependent differentiation (Schmidhauser et al., 1990). Thus, it can be concluded that neither cell–cell interactions, nor morphological polarization, are necessary for basement membrane-directed induction of  $\beta$ -casein expression.

#### *Basement Membrane Can Direct Differentiation in the Absence of Serum or Other Growth Factors*

There is an increasing awareness that growth and differentiation factors can be bound by ECM and be presented to cells

Table III. Effect of Substratum on Differentiation of Single Cells or Cell Clusters

Experiment	Substratum	Percentage of $\beta$ -casein staining			Total
		Single cells	2 cell clusters	$\geq 4$ cell clusters	
1	EHS matrix	54 (307)	86 (102)	96 (94)	(590)
	collagen I	4 (369)	34 (70)	74 (50)	(557)
2	EHS matrix	71 (98)	94 (34)	95 (22)	(180)
	collagen I	7 (175)	51 (69)	74 (42)	(320)
3*	EHS matrix	55 (114)	72 (54)	98 (45)	(260)
	collagen I	7 (81)	23 (31)	75 (25)	(155)

\* Cells cultured in whole mounts were analyzed in this experiment.

in this form (Bradley and Brown, 1990; Klagsbrun, 1990; Rathjen et al., 1990; Ruoslahti and Yamaguchi, 1991). It was therefore necessary to test whether factors potentially present in EHS matrix (Vigny et al., 1988) or in serum contributed to milk production. If already active, or if activated in culture, such factors could participate in the control of mammary differentiation. SDS-PAGE detectable low molecular weight proteins were removed from EHS matrix using

an ammonium sulfate precipitation protocol shown previously to deplete the matrix of factors such as TGF $\alpha$ , TGF $\beta$ , and EGF (Taub et al., 1990). Primary mammary cells formed bona fide spherical structures on this substratum (not shown) and synthesized high levels of milk proteins (Fig. 8) in the absence of serum. Cryosections with similar immunofluorescence profiles to those in Fig. 2 revealed that  $\beta$ -casein was secreted vectorially into alveolar lumina. Fur-

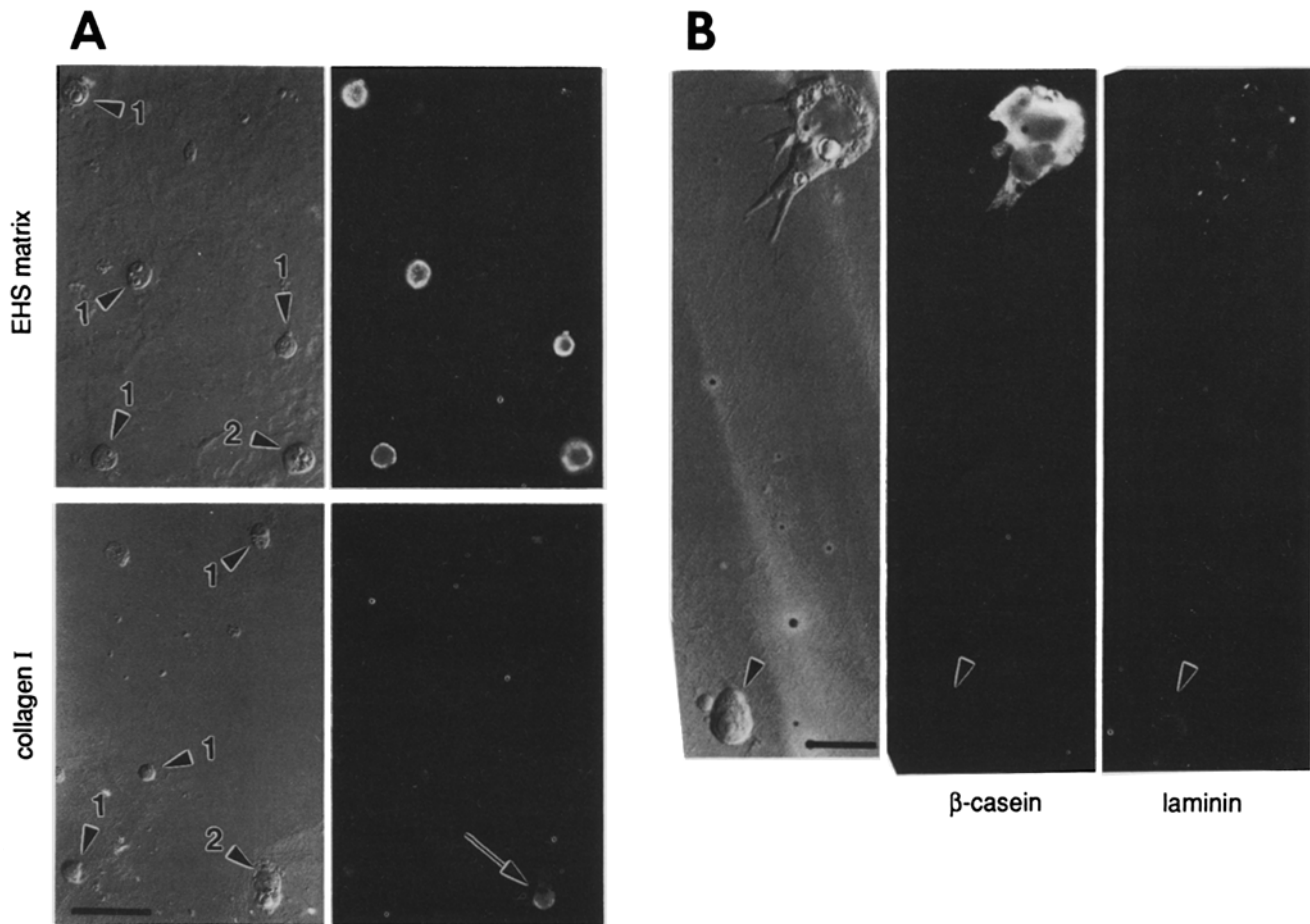
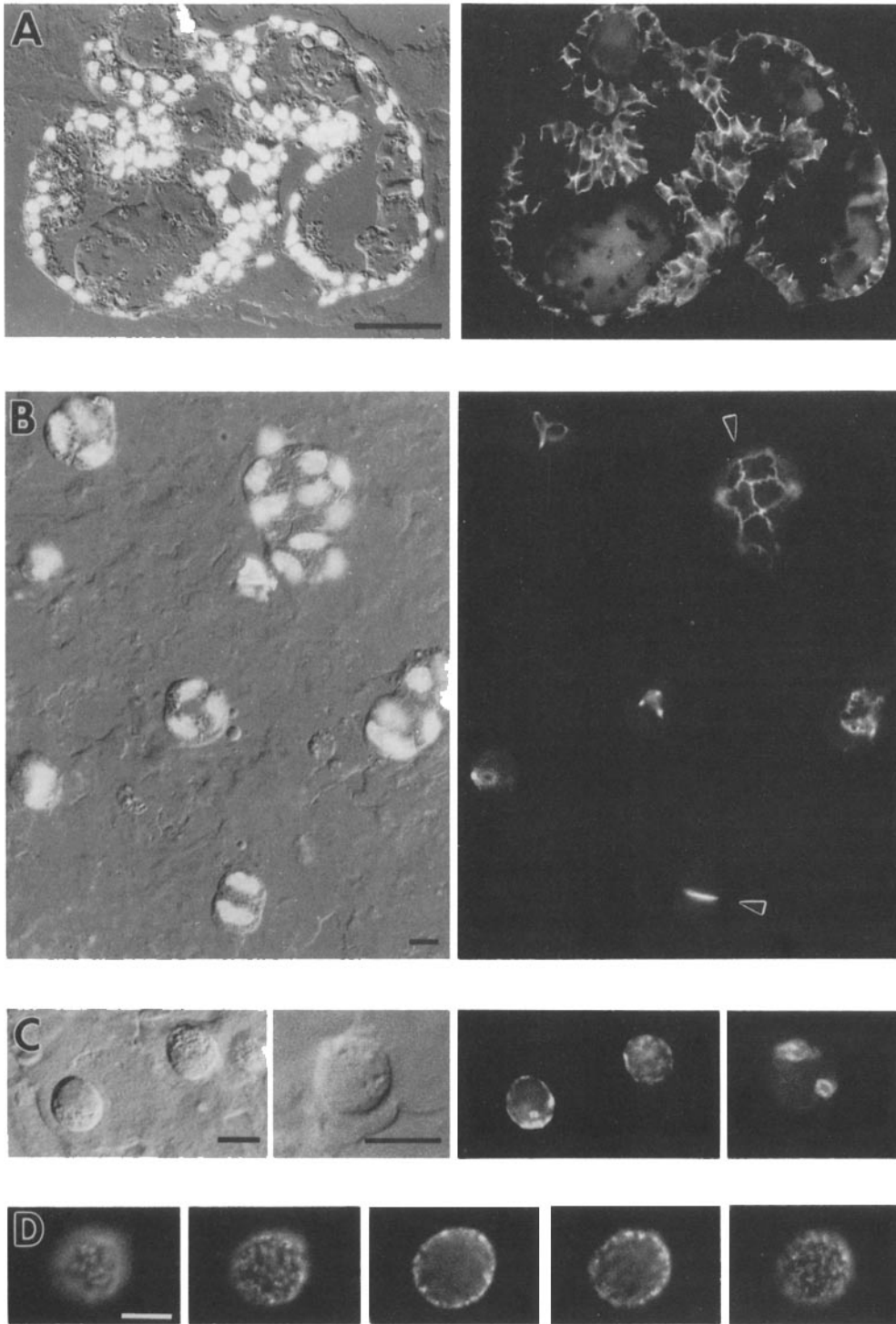


Figure 5. Single cells cultured inside collagen I do not express  $\beta$ -casein. (A) 25- $\mu$ m cryosections of cells embedded inside either EHS matrix or collagen I were stained for their ability to express  $\beta$ -casein. Although the majority of cells inside the basement membrane gel were positive, single cells inside collagen I were not induced to express this gene; positives only existed in cell aggregates, and even then the staining was much weaker than that in EHS matrix embedded cells (arrow). These fluorescence micrographs were exposed for equivalent amounts of time. This number of nuclei in each cluster are indicated. (B) A section of cells cultured inside collagen I was stained with antibodies for  $\beta$ -casein and laminin. At the top of the field, laminin stippling is visible in close association with a casein-expressing two cell cluster. At the bottom is a single cell (arrowhead) that expressed neither laminin nor  $\beta$ -casein. Bars: (A) 50  $\mu$ m; (B) 20  $\mu$ m.



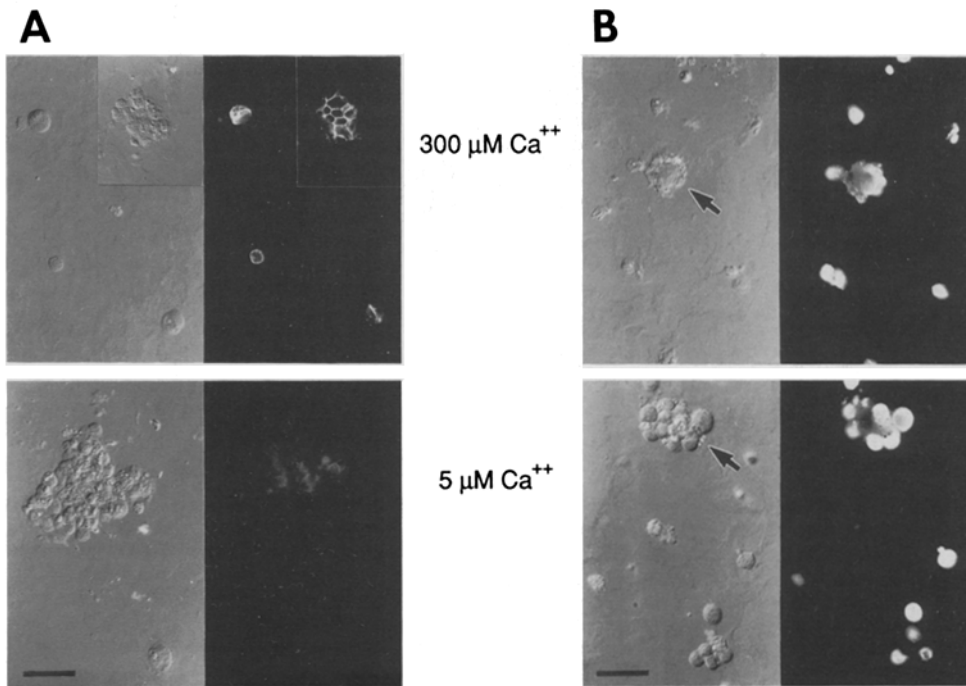
**Figure 6.** Distribution of E-cadherin on mammary cells in EHS matrix culture. (A) The highly polarized epithelium of cells cultured as 'alveoli' on a reconstituted basement membrane segregated E-cadherin mostly to lateral cell surfaces. Here, a 4- $\mu\text{m}$  cryosection of primary mammary cells cultured on EHS matrix for six days was stained by indirect immunofluorescence with ECCD-2, a mAb specific for E-cadherin. In the Normarski image, DAPI-stained nuclei are visible. Compare the morphology of this structure with that shown in Fig. 2. (B-D) 25- $\mu\text{m}$  cryosections of embedded mammary cells were stained by immunofluorescence with ECCD-2. (B) Note that E-cadherin accumulated at regions of cell-cell interaction (arrowheads). (C) Single cells show patching of E-cadherin to several sites around each cell. (D) Such patching is seen more clearly in another view of a single cell, photographed at different levels of focus. Bars: (A) 50  $\mu\text{m}$ ; (B-D) 10  $\mu\text{m}$ .

thermore, cells on plastic dishes that had not been exposed to growth factors for three days, and then suspension cultured in factor-depleted EHS matrix in the absence of serum for a further six days, were still induced to express  $\beta$ -casein; indeed, in one experiment 93% of single cells and 100% of cell clusters were casein-positive under these conditions (compare with Table I).

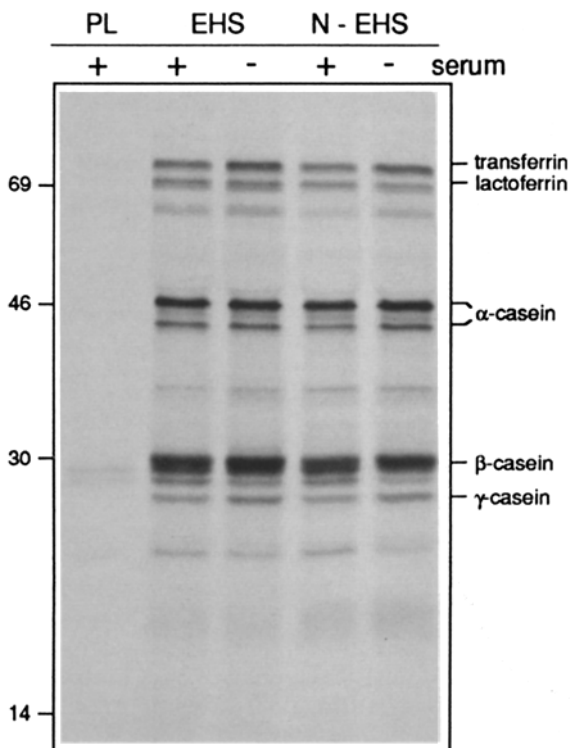
These results suggest that apart from a requirement for lactogenic hormones, biochemical differentiation of mammary cells is independent of signals from growth factors, although

it is not yet possible to design experiments that rule out involvement of autocrine growth factors which may be ECM bound. However, if factors are produced endogenously they are induced to do so by the basement membrane matrix, since collagen I is not effective at causing single cells to function. The results presented below demonstrate further that basement membrane itself provides direct signals for tissue-specific gene expression, since ECM receptors are involved in this process.





**Figure 7.** Single cells can be induced to express  $\beta$ -casein even in  $5 \mu\text{M Ca}^{2+}$ . (A) E-cadherin was localized to regions of contact in mammary cells suspension-cultured in EHS matrix, but only in normal medium with  $300 \mu\text{M Ca}^{2+}$ . In medium containing only  $5 \mu\text{M Ca}^{2+}$  it was almost completely absent, even in large clusters of cells. The fluorescence micrographs were exposed for equivalent amounts of time. (B) In contrast  $\beta$ -casein is readily visible, even at low concentrations of  $\text{Ca}^{2+}$ . In two experiments, the percentage of positive single cells was similar under both conditions. Note that while the cell clusters inside normal EHS matrix coalesced, those cultured in  $5 \mu\text{M Ca}^{2+}$  often failed to do so (arrows; these clusters each contained more than 10 cells). Bars,  $50 \mu\text{m}$ .



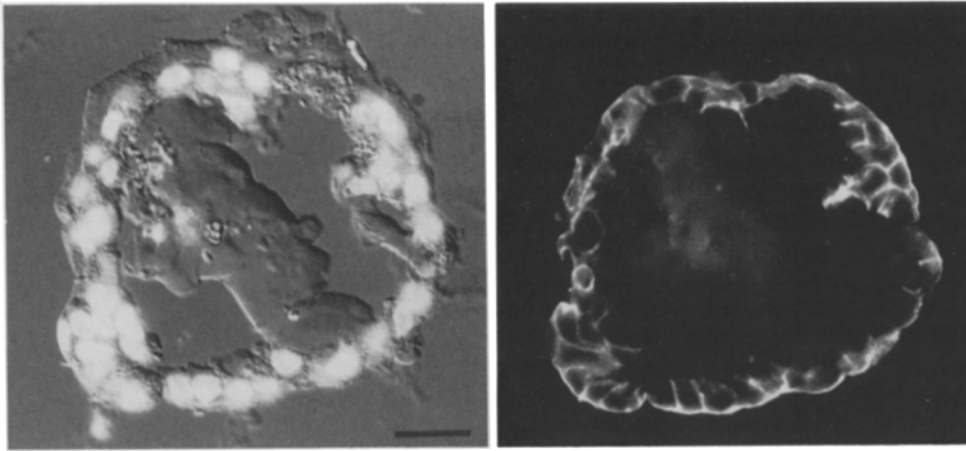
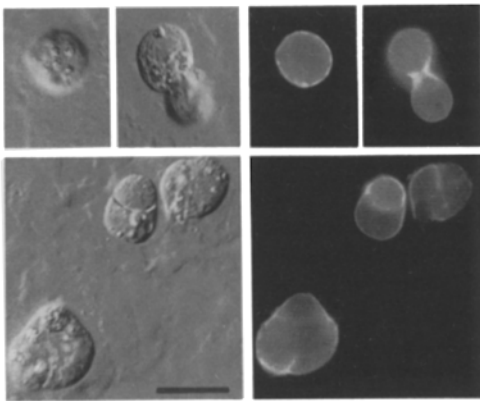
**Figure 8.** Milk protein expression is induced by basement membrane in the absence of matrix-associated soluble factors. In the presence of lactogenic hormones, growth factor-depleted EHS matrix (N-EHS) was permissive for the expression of a full range of milk proteins by primary mammary epithelial cells. Differentiation was not adversely affected by the complete absence of serum. Here, cell fractions, including material vectorially secreted into the alveolar lumina, were isolated from pulse-labelled cultures, immunoprecipitated with a milk-specific polyclonal antiserum, and the milk proteins were separated by SDS-PAGE. Size markers (in kD) are indicated.

### *Integrins Are Involved in the Control of Mammary Differentiation*

Little is known about the types of integrins in mammary cells. One receptor for the major basement membrane component laminin is the  $\alpha_6$  integrin heterodimer (Sonnenberg et al., 1990), which in mammary gland (not shown) and in cultured alveoli (Fig. 9 A) is predominantly basal. In EHS matrix suspension culture, this ECM receptor was localized uniformly around the periphery of single cells and clusters, indicative of an interaction with the surrounding substratum (Fig. 9 B). In monolayer cultures on plastic dishes there was faint intercellular staining although none was seen basally (not shown), reflecting the absence of a basement membrane (Streuli and Bissell, 1990).

An  $\alpha_6$ -specific mAb (GoH3), shown previously to block binding of primary mammary epithelial cells (not shown) and other cell types to laminin (Sonnenberg et al., 1988, 1990; Hall et al., 1990; Lotz et al., 1990), had no effect on the ability of single cells to produce  $\beta$ -casein (Table IV, experiment 1). Since we did not know which specific integrins are involved in the interaction between mammary cells and ECM, we took advantage of an antibody that could broadly block such interactions via  $\beta_1$  integrins (Tomaselli et al., 1987, 1988).

When single mammary cells were cultured in the presence of such an anti-integrin antibody (anti ECM-R; Knudsen et al., 1981; Damsky et al., 1982),  $\beta$ -casein synthesis was reduced dramatically (Table IV, experiments 2-4). A few single cells and individual cells within clusters escaped this block, possibly due to antibody accessibility; however, even those cells that were positive, stained weakly in comparison to those in the control experiments. In single, embedded CID-9 cells,  $\beta$ -casein synthesis was blocked completely (Table IV, experiment 5), showing that integrin-mediated signalling was not restricted to secondary cultures of mammary

**A****B**

**Figure 9.**  $\alpha_6$  Integrin in mammary epithelial cells. (A) The basal distribution of  $\alpha_6$  integrin is evident in this 4- $\mu\text{m}$  cryosection of primary mammary cells cultured for six days on EHS matrix. The section was stained by indirect immunofluorescence with GoH3, a mAb specific for  $\alpha_6$  integrin. This integrin was also localized to some extent at regions of cell-cell contact. Compare the morphology of this alveolar structure with that shown in Fig. 2. (B)  $\alpha_6$  integrin was located at peripheral regions of mammary cells embedded inside EHS matrix. Here, 25- $\mu\text{m}$  cryosections were stained by immunofluorescence with GoH3. Bars, 20  $\mu\text{m}$ .

epithelia, but applied also to functional cell strains. The anti ECM-R antibody also inhibited  $\beta$ -casein expression in the few positive single cells cultured in the collagen I gel system. In contrast, antibodies that bound to and interfered with the normal activity of other surface molecules such as E-cadherin (ECCD-1; Yoshida-Noro et al., 1984) had no effect on the expression of  $\beta$ -casein in suspension cultured cells (Table IV, experiments 4 and 5). Since both this and the GoH3 antibodies failed to interfere with differentiation, their presence within the EHS matrix (and indeed, on the surface of embedded cells) was confirmed by immunostaining (not shown). Finally, to show that the anti ECM-R antibody was specific and did not disrupt the normal pattern of cell-cell contact in clusters, the distribution of E-cadherin was examined and was found to be similar to that in controls.

### Discussion

Two major conclusions can be derived from this work. First, the basement membrane has a central role in controlling tissue-specific gene expression in mammary epithelial cells, and integrins are involved in this signal transduction. Second, cell-cell interaction is not necessary for the synthesis of  $\beta$ -casein in the presence of a basement membrane, but such interaction enhances  $\beta$ -casein production in a stromal matrix.

### Basement Membrane Directs $\beta$ -Casein Expression

Evidence that basement membrane itself is sufficient to direct expression of  $\beta$ -casein in the presence of lactogenic hormones comes from two observations. (a) Single and separated mammary cells, which have lost the capacity to synthesize milk proteins after being cultured on plastic dishes, could be induced to reexpress this gene in the presence of a laminin-rich, basement membrane matrix, but not in the presence of other physiological substrata such as collagen I. ECM-bound growth factors do not appear to be the determining element in this process; support for this comes from our work with collagen I cultures where  $\beta$ -casein is expressed only if an endogenously produced basement membrane is deposited after floatation of the collagen I gel (Streuli and Bissell, 1990). However, our studies do not preclude a role for autocrine, ECM-bound growth factors; (b) an anti-integrin antiserum that interferes with the cell-ECM interaction blocks biochemical differentiation in mammary epithelial cells. The antibody used in this study is known to block attachment of cells to laminin, collagen IV, and fibronectin substrata by interfering with  $\beta_1$  integrin function (Tomaselli et al., 1987, 1988), indicating that this class of ECM receptors transduces the signals controlling  $\beta$ -casein expression.

It is now clear that a major control point in the expression of  $\beta$ -casein occurs at the transcriptional level. Convincing

**Table IV. Effect of Antibodies on Differentiation of Single Cells or Cell Clusters Cultured for Six Days inside EHS Matrix**

Experiment	Antibody	Percentage of $\beta$ -casein staining			Total
		Single cells	2 cell clusters	>4 cell clusters	
1	None	86 (106)	94 (51)	100 (14)	(200)
	GoH3	83 (83)	85 (40)	92 (13)	(162)
2	None	51 (169)	84 (45)	91 (33)	(281)
	Anti ECM-R <sup>‡</sup>	114(187)	17 <sup>†</sup> (18)	65 <sup>†</sup> (23)	(240)
3	None	71 (98)	94 (34)	95 (22)	(180)
	Anti ECM-R	9 <sup>†</sup> (96)	15 <sup>†</sup> (20)	75 <sup>†</sup> (8)	(133)
4	Goat serum	77 (62)	83 (29)	86 (7)	(119)
	ECCD-1 <sup>§</sup>	74 (62)	88 (25)	100 (5)	(118)
	Anti ECM-R	16 <sup>†</sup> (122)	23 <sup>†</sup> (64)	43 <sup>†</sup> (7)	(205)
5*	None	27 (205)	39 (105)	89 (104)	(508)
	ECCD-1	24 (108)	32 (53)	79 (56)	(268)
	Anti ECM-R	0 (210)	0 (62)	30 <sup>†</sup> (113)	(470)

\* CID-9 cells were used in this experiment.

<sup>‡</sup> Anti ECM-R is a pan-specific anti  $\beta_1$  integrin antibody.

<sup>§</sup> ECCD-1 is a function-blocking anti E-cadherin antibody; in our hands, ECCD-1 caused cells cultured as monolayers on plastic dishes to separate from each other within a few hours.

<sup>†</sup> Clusters were scored positive even if only one of the group stained; the level of staining in single cells was often lower than in the controls.

evidence exists for the presence of an ECM-dependent regulatory element in the upstream region of the  $\beta$ -casein gene (Schmidhauser et al., 1990; and unpublished results). Thus, the consequence of an ECM-integrin interaction in these cells is an intracellular cascade of events leading to a dramatic increase in the expression of tissue-specific genes.

Our results widen the spectrum of biological processes that are integrin mediated (Albeda and Buck, 1990; Ruoslahti and Giancotti, 1990). Two other examples of altered gene expression resulting from signal transduction through integrins have been documented (Adams and Watt, 1989; Werb et al., 1989). In addition, many developmental events in invertebrates (Leptin et al., 1989; Zusman et al., 1990; Volk et al., 1990) and in vertebrates involve integrins. These include the migration of neural crest cells (Bronner-Fraser, 1986) and myoblasts (Jaffredo et al., 1988), myogenesis (Menko and Boettiger, 1987; Volk et al., 1990), trophoblast outgrowth (Sutherland et al., 1988), kidney development (Sorokin et al., 1990), and the outgrowth of neurites in developing neural tissue (Reichardt et al., 1989).

A number of integrins are candidates for the signal transduction process; several different integrin heterodimers for example, are receptors for epitopes contained within basement membranes (Mercurio, 1990). Despite the basal distribution of  $\alpha_6$  integrin in the mammary cultures, our results suggest that this integrin may not be responsible for basement membrane-mediated signalling; but given the fact that we have tested only one  $\alpha_6$  integrin antibody, we cannot rule out its involvement at this stage.  $\alpha_3\beta_1$  integrin is an alternative possibility since it is recognized by the anti ECM-R antiserum in PC 12 cells (Tomaselli et al., 1987; Reichardt et al., 1989), and is expressed in our mammary epithelial cultures (unpublished data). However, the precise identity of the signalling molecule will have to await the production of additional rodent-specific anti-integrin reagents.

### ***$\beta$ -Casein Expression Is Independent of Cell-Cell Contact***

The ability to express  $\beta$ -casein in cells that were separated from each other, and suspended within a basement membrane matrix, implies that some features of differentiation are independent of cell-cell contact. This conclusion is supported by our experiments in which casein production: (a) could still be induced in the presence of only 5  $\mu$ M  $Ca^{2+}$ ; but (b) was largely inhibited by anti integrin antibodies even in clusters of cells, although contact molecules such as E-cadherin were located normally. In all these studies cells were plated on average at least five cell diameters apart, and at both this and at lower cell densities  $\beta$ -casein was synthesized.

In one other system, that of keratinocytes, terminal differentiation of individual cells can occur in the absence of cell-cell contact (Green, 1977; Watt et al., 1988). In this case, however, it is the detachment from the substratum and a reduction in the ability of integrins to bind certain ECM components that triggers a phenotypic response (Adams and Watt, 1990). Thus, the situation in stratified epithelium contrasts with that in a glandular tissue such as mammary gland.

Cell-cell contact is required, however, for full differentiation in secretory epithelial monolayers. Indeed, contact is essential for the onset of morphological and functional polarity (Rodriguez-Boulan and Nelson, 1989; Gumbiner, 1990), and has been implicated previously for mammary differentiation. Cells cultured on collagen I gels for example, only synthesize milk proteins when the gels are floated into the medium, an event that triggers profound changes in cytostructure and induces a high degree of cell-cell interaction (Emerman and Pitelka, 1977). Although this results in the facility for secretion, it also results in the deposition of an intact basement membrane (Streuli and Bissell, 1990). As is

the case with MDCK cells (Wang et al., 1990), this would contribute to full polarization of the monolayer. From the data presented in this paper, we now believe that it is this basement membrane which provides the critical signals for biochemical differentiation in mammary cells. Accordingly, in collagen I suspension culture where there is no exogenous basement membrane, essentially only those cells in clusters are able to express  $\beta$ -casein. Thus, in a stromal environment cell-cell contact does appear to be necessary for function, and indeed, the cell-associated laminin (and other ECM components) in such clusters may be relevant to the observed tissue-specific gene expression.

### ***$\beta$ -Casein Expression Is Independent of Morphological Polarity***

Single cells embedded inside the basement membrane matrix remain rounded and do not appear to be polarized at the morphological level.  $\beta$ -casein is distributed uniformly and does not show any apical-basal segregation. Neither E-cadherin nor  $\alpha_6$  integrin appear to be directed to polar (lateral or basal) regions; indeed the latter is distributed evenly around the cell surface. Since the formation of a polarized epithelium depends upon the segregation of plasma membrane into distinct regions that define apical and basal surfaces (Rodríguez-Boulan and Nelson, 1989; Gumbiner, 1990), our results indicate that the expression of tissue-specific genes such as  $\beta$ -casein can be dissociated functionally from the establishment of morphological polarity.

Two further lines of evidence support this conclusion. Firstly, mammary cells cultured as flat monolayers on plastic dishes establish certain elements of polarity, yet such cultures largely lose their capacity to express milk proteins: E-cadherin, required for polarization of basal-lateral membranes (Nelson et al., 1990), and the Na<sup>+</sup>, K<sup>+</sup>-ATPase (McNeill et al., 1990) were both present on the lateral cell surfaces of our primary monolayer cultures; ZO-1, essential for the formation of tight junctions (Siliciano and Goodenough, 1988), appeared at the apical region between adjacent cells (our unpublished data); further, an actin cytoskeleton with associated proteins such as PAS-O could be organized apically in a mammary cell line cultured on plastic dishes (Parry et al., 1990). Secondly, in 5  $\mu$ M Ca<sup>2+</sup> medium, E-cadherin was not evident in cultures of cells embedded within EHS matrix. These cells therefore lacked polarity even within the cell clusters, yet  $\beta$ -casein was still synthesized.

### ***Formation of a Fully Functional Epithelium***

Our results allow us to propose a hierarchical model for the establishment and maintenance of a fully differentiated mammary epithelium. Interactions between epithelial cells provide signals for both the correct organization of intracellular cytostructure and the deposition of a basement membrane. In turn, the basement membrane contributes to the generation of a fully polarized, and tight, epithelium, enclosing a lumen into which milk can be secreted. Ultimately, the basement membrane itself provides molecular signals that regulate tissue-specific gene expression and, in mammary gland, allows the synthesis and secretion of  $\beta$ -casein.

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